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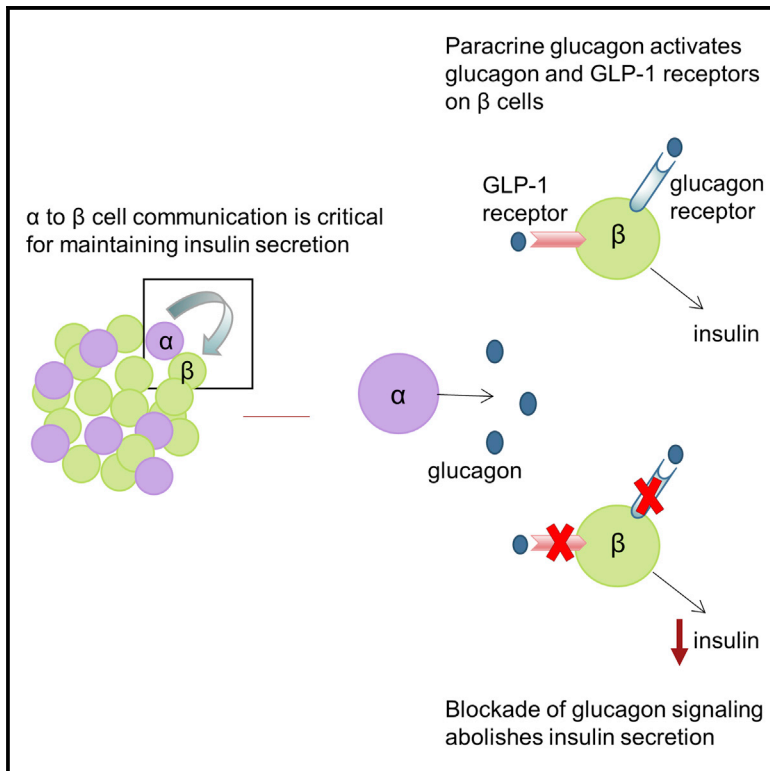
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Insulin Secretion Depends on Intra-islet Glucagon Signaling

Graphical Abstract



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In Brief

Glucose-stimulated insulin secretion can be regulated by glucagon and GLP-1 receptors on paracrine β -cells. Svendsen et al. find that complete blockade of glucagon signaling in islets severely limits insulin secretion but establish that paracrine glucagon signaling involves both the glucagon and GLP-1 receptors.

Highlights

- Paracrine glucagon actions are required for maintenance of normal insulin secretion
- Glucagon signaling in islets involves activation of glucagon and GLP-1 receptors
- Beta cell GLP-1 receptors are activated by local glucagon in a paracrine manner



Insulin Secretion Depends on Intra-islet Glucagon Signaling

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SUMMARY

The intra-islet theory states that glucagon secretion is suppressed when insulin secretion is stimulated, but glucagon's role in intra-islet paracrine regulation is controversial. This study investigated intra-islet functions of glucagon in mice. We examined glucagon-induced insulin secretion using isolated perfused pancreata from wild-type, GLP-1 receptor (GLP-1R) knockout, diphtheria toxin-induced proglucagon knockdown, β cell-specific glucagon receptor (Gcgr) knockout, and global Gcgr knockout (*Gcgr*^{−/−}) mice. We found that glucagon stimulates insulin secretion through both Gcgr and GLP-1R. Moreover, loss of either Gcgr or GLP-1R does not change insulin responses, whereas combined blockage of both receptors significantly reduces insulin secretion. Active GLP-1 is identified in pancreatic perfusate from *Gcgr*^{−/−} but not wild-type mice, suggesting that β cell GLP-1R activation results predominantly from glucagon action. Our results suggest that combined activity of glucagon and GLP-1 receptors is essential for β cell secretory responses, emphasizing a role for paracrine intra-islet glucagon actions to maintain appropriate insulin secretion.

INTRODUCTION

Glucose homeostasis is primarily regulated by the hormones insulin and glucagon, secreted from the pancreatic islets of Langerhans. Although insulin produced by β cells is the blood glucose-lowering hormone, glucagon secreted from α cells acts as the major counter-regulatory hormone to insulin and is important for maintaining normal glucose levels. Conversely, glucagon secretion is regulated by glucose, being inhibited during hyperglycemia and stimulated during hypoglycemia, resulting in stimulation of hepatic glucose output (Gromada et al., 2007). Disturbed control of α cell function is characteristic of type 2 diabetes and, importantly, results in hyperglucagonemia, which contributes to hyperglycemia (Kahn, 2003; Kazda et al.,

2016; Knop et al., 2007). The development of hyperglucagonemia would be consistent with the so-called intra-islet hypothesis stating that glucagon secretion undergoes inhibition from insulin, which, in conditions with decreased insulin secretion or α cell insulin resistance, would lead to reduced suppression of glucagon secretion (Banerjee et al., 2002; Hope et al., 2004; Meier et al., 2006). On the other hand, glucagon is also known as an efficacious stimulator of insulin secretion, and the glucagon receptor is located on both pancreatic β and δ cells (Adriaenssens et al., 2016), suggesting that glucagon might have direct effects on insulin and somatostatin secretion.

In this study we investigate mechanisms regulating intra-islet communication, focusing on the role of glucagon in control of insulin secretion using the perfused mouse pancreas, a model that allows investigations of the paracrine relationships within intact islets. We employed a range of complementary genetic and pharmacological approaches to disrupt local glucagon signaling. Thus, to acutely eliminate glucagon secretion from α cells, we used diphtheria toxin (DT)-induced destruction of proglucagon-producing α cells (Pedersen et al., 2013). For estimation of the importance of glucagon receptors (Gcgrs) in β cells, we used a mouse line with inducible Gcgr knockout exclusively in β cells (*Gcgr* ^{β cell−/−}) as well as mice with global knockout of the Gcgr (*Gcgr*^{−/−}) (Gelling et al., 2003). The functional importance of the glucagon-like peptide-1 receptor (GLP-1R) was investigated using *Glp1r*^{−/−} mice (Scrocchi et al., 1996) as well as the GLP-1R antagonist exendin(9-39) (Ex9).

RESULTS

Exogenous Glucagon Stimulates Insulin Secretion through the Glucagon and GLP-1 Receptors

In pilot studies using the perfused mouse pancreas, we observed that glucagon was only able to stimulate insulin secretion at elevated (12 mM) glucose levels, whereas no response was observed at 3.5 mM glucose (Figures S1A and S1B). Thus, the effect of exogenous glucagon was examined further only at high glucose levels. Infusions of glucagon (0.1–10 nM) stimulated insulin secretion in a dose-dependent manner with 2.0- to 3.7-fold increases (Figure 1). Glucagon infusions (1–10 nM) were still able to increase insulin secretion, albeit from a lower baseline, in the presence of the co-administered GLP-1R



antagonist Ex9 (Figures 1A and 1C) or in perfused pancreata from *Glp1r*^{-/-} mice (Figures 1B and 1D). Pancreata from mice with selective inactivation of the Gcgr only in β cells (*Gcgr* ^{β cell-/-}) showed similar levels (versus control) of insulin secretion in response to 1–10 nM glucagon (Figures 1E and 1G).

On the other hand, pancreata from mice with global Gcgr knockout (*Gcgr*^{-/-} mice) only increased insulin secretion in response to 10 nM glucagon and with a submaximal response relative to the control group, whereas lower doses of glucagon had no effect on insulin secretion (Figures 1F and 1H), consistent with the very high local concentrations of glucagon (see below). Combining blockade of glucagon and GLP-1 receptor by simultaneous addition of Ex9 in perfusions of *Gcgr*^{-/-} mice completely abolished the insulin responses to glucagon and glucose (Figures 1F and 1H).

Disruption of Endogenous Glucagon Signaling Reduces Insulin Secretion

Different methods of selective or complete disruption of glucagon signaling were used in four different mouse strains to investigate the effect of endogenous glucagon on insulin secretion. In all control mice, switching from low (3.5 mM) to high (12 mM) glucose led to an ~4.5-fold increase in insulin ($p < 0.05$) and a simultaneous drop in glucagon secretion (~3-fold, $p < 0.05$) (Figure 2). Secretion of hormones was further stimulated by arginine infusion, except for insulin secretion at low glucose levels.

To investigate insulin secretion in islets with depletion of glucagon from α cells, we used DT-Gcg mice. Total glucagon output from these pancreata was significantly reduced compared with control mice, reflecting successful ablation of α cell mass ($p < 0.001$; Figure 2A). Insulin responses (Figures 2B and 2G) to increased glucose concentrations and arginine were significantly reduced after α cell ablation and reduction of islet glucagon secretion ($p < 0.01$). DT had no effect in wild-type mice (Figure S2).

Mice with knockout of the Gcgr exclusively in β cells showed a pattern of glucagon and insulin secretion in response to glucose and arginine similar to that obtained using pancreata from cre control mice (Figures 2C, 2D, and 2H).

In pancreata from *Gcgr*^{-/-} mice (Figure 2E), glucagon secretion was markedly elevated by up to 26-fold compared with littermate controls. Insulin secretion was significantly stimulated in *Gcgr*^{-/-} mice by infusion of arginine and high glucose and increased compared with *Gcgr*^{+/+} mice. To determine the contribution of the GLP-1R, we infused Ex9, which significantly reduced insulin secretion in response to high glucose and arginine in *Gcgr*^{-/-} mice compared with experiments without Ex9, resulting in an ~3-fold reduction in total insulin output ($p < 0.001$). In control mice, Ex9 tended to reduce the insulin response to high glucose levels, but arginine induced a similar increase in insulin levels in the presence or absence of Ex9 (Figures 2F and 2I).

The GLP-1 Receptor Antagonist Exendin(9-39) Inhibits Both GLP-1- and Glucagon-Mediated cAMP Responses via the GLP-1 Receptor

The ability of glucagon and GLP-1 to activate mouse Gcgr and GLP-1R was examined in non-islet cells transiently expressing the receptors. Glucagon and GLP-1 activated their respective

receptors with potencies in the sub-nanomolar range (half maximal effective concentration [EC_{50}] of 0.23 ± 0.09 nM and 0.15 ± 0.11 nM, respectively) (Figures 3A and 3B). Furthermore, glucagon acted as a weak agonist on the GLP-1R ($EC_{50} = 36.4 \pm 0.22$ nM), approaching the maximal response (E_{max}) of GLP-1 on the GLP-1R (~88% activation compared with GLP-1) (Figure 3A). In contrast, only glucagon, but not GLP-1, activated the Gcgr (Figure 3B), consistent with previous findings (Jorgensen et al., 2007).

We next verified the relative selectivity of Ex9 for the GLP-1R. As expected, increasing concentrations of Ex9 resulted in a decrease in GLP-1-induced cyclic AMP (cAMP) accumulation via the GLP-1R with an half maximal inhibitory concentration (IC_{50}) value of 12 ± 0.47 nM (Figure 3C). Ex9 was also able to abrogate glucagon-mediated cAMP responses via the GLP-1R with the same IC_{50} value of 12 ± 0.44 nM (Figure 3C). It was, however, not able to inhibit glucagon-stimulated cAMP responses mediated through the Gcgr (Figure 3D). Addition of Ex9 reduced the GLP-1 and glucagon-mediated cAMP levels below the basal levels, indicating that Ex9 also acts as an inverse agonist on the GLP-1R, as suggested previously (Serre et al., 1998).

Exendin 9-39 Reduces Insulin Secretion from the Perfused Mouse Pancreas

We further evaluated the direct effect of Ex9 on basal insulin secretion by perfusion with Ex9. Addition of Ex9 significantly reduced insulin secretion in pancreata from control and *Gcgr*^{-/-} mice at high glucose levels, whereas no significant effect was observed in *Glp1r*^{-/-} mice, confirming that Ex9 is specific for the GLP-1 receptor (Figures 4A and 4B).

Pancreatic GLP-1 Immunoreactivity

Considerable evidence suggests that, under some circumstances, the pancreas produces immunoreactive and bioactive GLP-1 (Marchetti et al., 2012; Taylor et al., 2013), which may contribute to a local incretin axis (Chambers et al., 2017). Accordingly, we evaluated whether fully processed active GLP-1 (7-36_{NH2}/37) is secreted from perfused pancreata of *Gcgr*^{-/-} and *Gcgr*^{+/+} mice (Figure 4C). Secretion of fully processed intact GLP-1 was very low in control mice (0–2 pM at low and high glucose) and often below the assay detection limit. Secretion of intact GLP-1 was significantly higher from *Gcgr*^{-/-} pancreata (5.5-fold in total output; Figure 4C). Simultaneous perfusion of the pancreas with a DPP4 inhibitor (valine-pyrrolidide) did not change the levels of active GLP-1 in control or *Gcgr*^{-/-} mice (Figure 4C), suggesting that DPP4-induced GLP-1 degradation is negligible in the perfused mouse pancreas.

DISCUSSION

The interplay between glucagon and insulin secretion has been the subject of many studies, and the prevailing concept reflects the “intra-islet hypothesis” that insulin inversely regulates glucagon secretion. This hypothesis would be consistent with the theory of core-to-mantle blood flow, with β cells reacting to changes first and mantle cells (α and δ cells) then responding to insulin (Bonner-Weir and Orci, 1982; Maruyama et al., 1984). However, other studies have suggested that blood flow rather

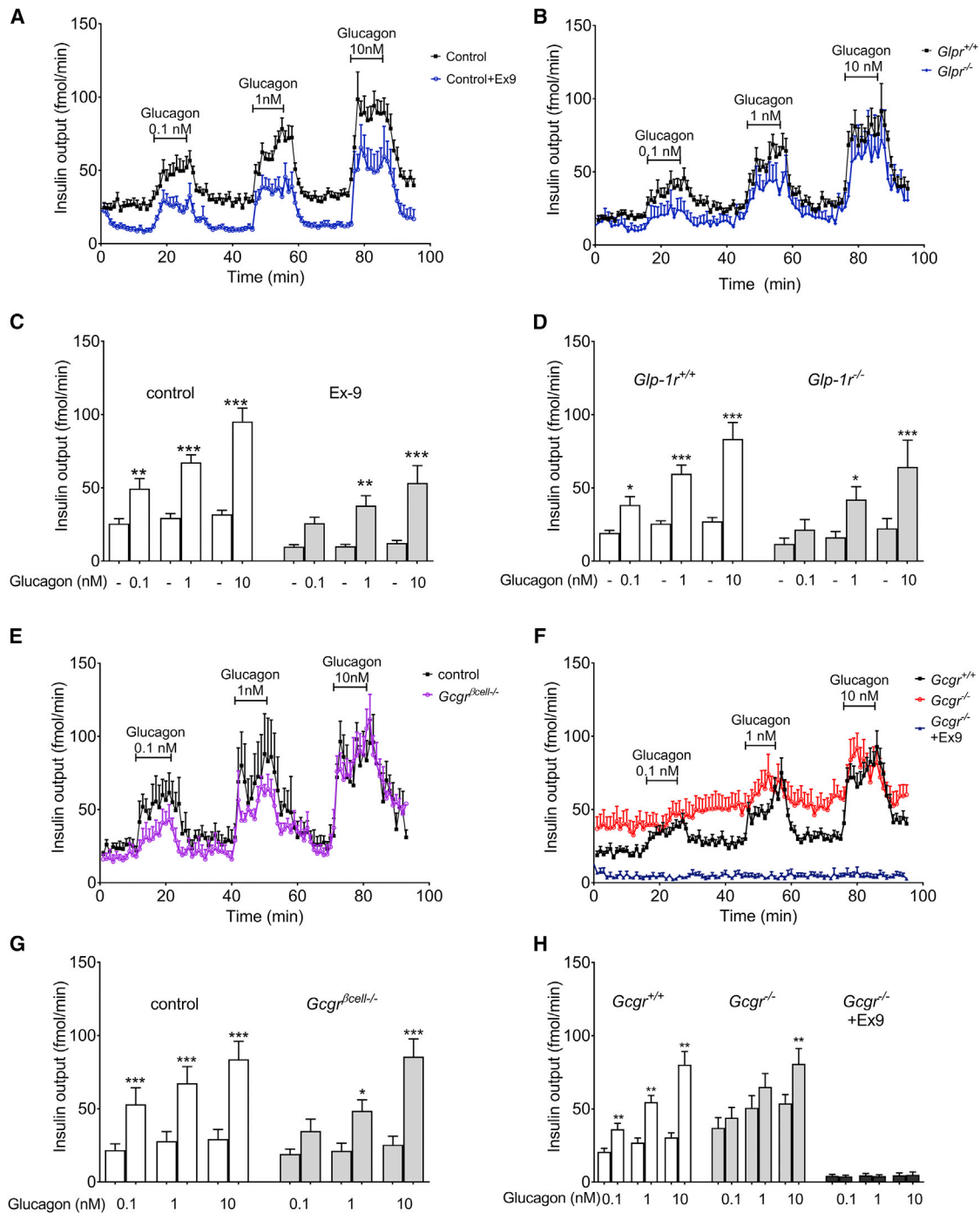


Figure 1. Exogenous Glucagon Infusions Stimulate Insulin Secretion through the Glucagon and the GLP-1 Receptor in Perfused Mouse Pancreata

(A, B, E, and F) Insulin secretion from perfused pancreata during infusion of glucagon (0.1–10 nM) at 12 mM glucose in control mice with or without Ex9 (A, n = 8), *Glp1r^{-/-}* mice (B, n = 6), *Gcgr^{βcell-/-}* mice (E, n = 5), and global *Gcgr^{-/-}* mice with or without Ex9 (F, n = 8).

(C, D, G, and H) Mean insulin output averaged over 10 min before and during addition of glucagon to perfused pancreata of control mice (C), *Glp1r^{-/-}* (D), *Gcgr^{βcell-/-}* (G), and *Gcgr^{-/-}* (H) mice. Significance was tested by 1-way ANOVA (repeated measures) comparing stimulated output versus respective basal output. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figure S1.

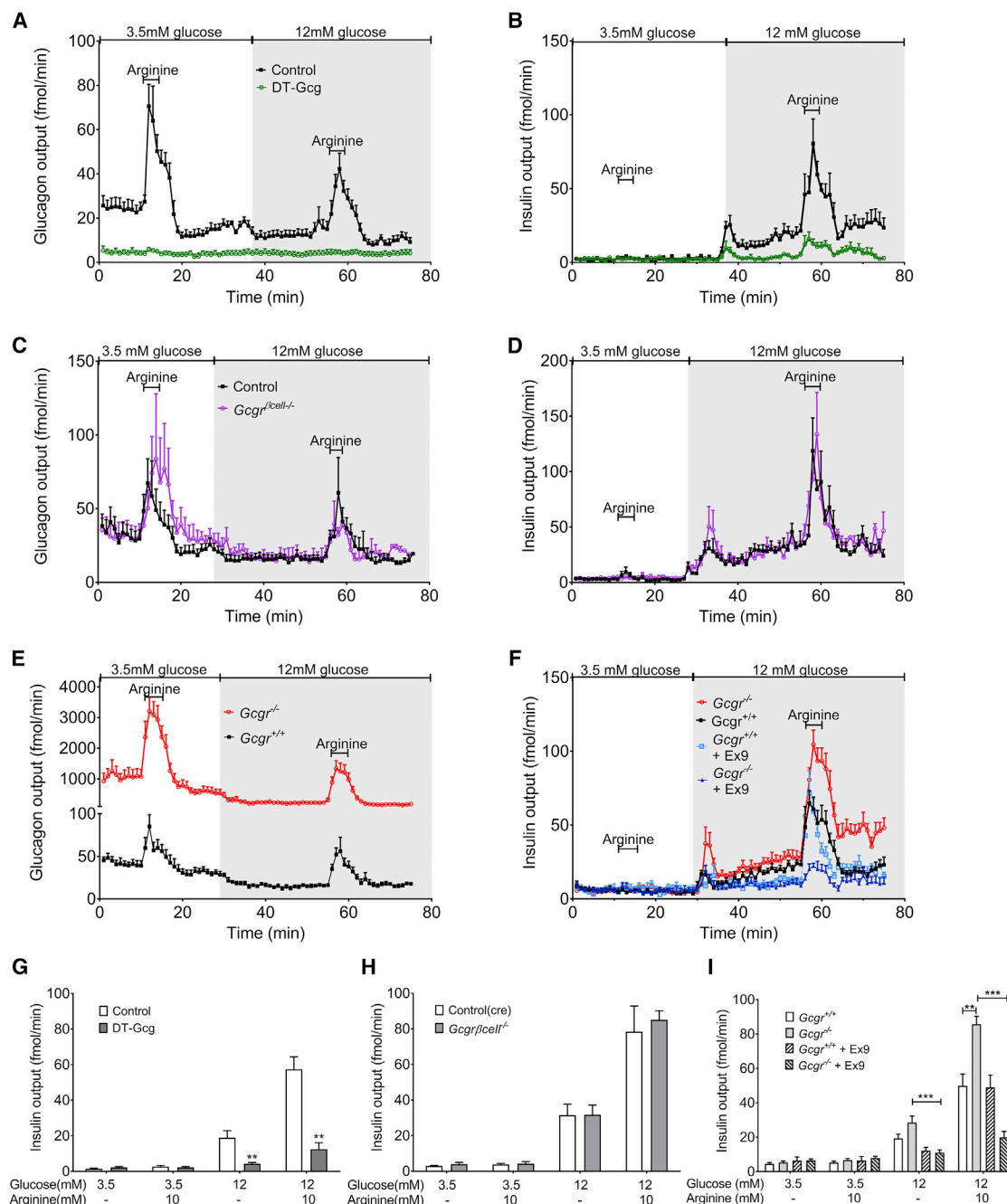


Figure 2. Effect of Disrupted Glucagon Signaling on Hormone Secretion from Perfused Mouse Pancreata

(A–F) Insulin and glucagon secretion from DT-Gcg (A and B, $n = 8$), $Gcgr^{\beta cell/-}$ (C and D, $n = 5$), and $Gcgr^{-/-}$ mice with or without addition of Ex9 (E and F; $n = 8$). (G–I) Mean insulin output averaged over 5 min before and during addition of glucose and arginine to perfused pancreata from control mice (G), $Gcgr^{\beta cell/-}$ mice (H), and $Gcgr^{-/-}$ mice (I). Significance was tested by 1-way ANOVA with *post hoc* Tukey modifications. Data are shown as mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$. See also Figure S2.

perfuses the islet from one side to the other regardless of cell type, thereby providing for paracrine actions of α and δ cell secretion on downstream β cells (Kharouta et al., 2009; Liu et al., 1993; Nyman et al., 2008). Exogenous glucagon is thought to stimulate insulin secretion, but endogenous glucagon is not

classically considered part of the paracrine β cell network (Kawamori et al., 2009; Leung et al., 2006; Ravier and Rutter, 2005). In this study, we revisited intra-islet paracrine relationships. An alternative approach to understanding intra-islet relationships is to use the isolated perfused pancreas preparations as

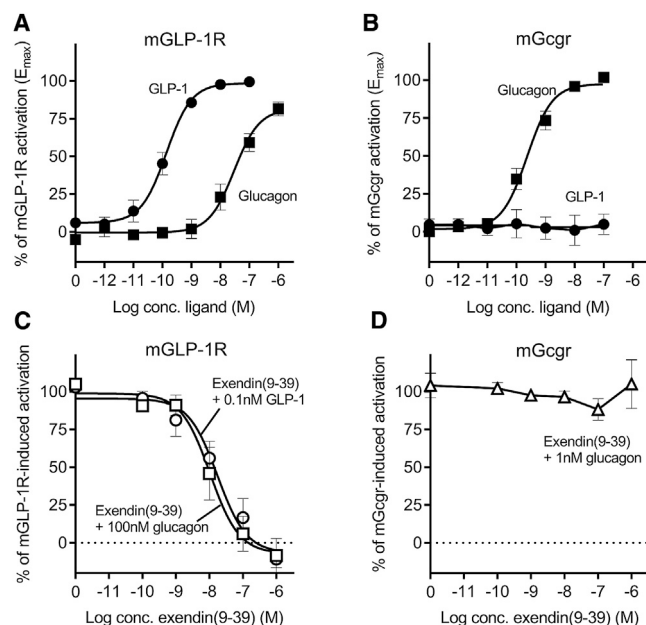


Figure 3. Activation and Inhibition of Glucagon and GLP-1 Receptors

(A and B) cAMP accumulation in COS-7 cells transfected with the mouse GLP-1 receptor (A) or glucagon receptor (B) after treatment with increasing concentrations of GLP-1 and glucagon. (C and D) cAMP accumulation after increasing concentrations of Ex9 in the presence of fixed concentrations of GLP-1 (C) or glucagon (C and D) in COS-7 cells transfected with the mGLP-1 receptor (C) and glucagon receptor (D). The dotted line represents basal receptor activity in the absence of agonist. Data are shown as mean \pm SEM, $n \geq 3$ independent experiments run in triplicates.

opposed to the often used isolated islet or *in vivo* studies. This technique allows functional interrogation of islet secretory activity without interference from other organs in the body, excluding systemic factors, elimination and/or degradation of hormones, etc., which often complicates interpretation of *in vivo* studies. Importantly, the perfused pancreas maintains its local cytoarchitecture and microvasculature, ensuring normal flux of both substrates and products to and from the various cell types.

Glucagon was described as an insulin secretagogue as early as 1965 (Samols et al., 1965). Our studies extend these findings by demonstrating that this is a direct effect mediated by distinct receptors in the mouse pancreas. Previous experiments on isolated β cells clearly show that glucagon is capable of directly stimulating insulin secretion, and β cells (and δ cells) have been reported to express Gcgrs (Adriaenssens et al., 2016; Huypens et al., 2000; Van Schravendijk et al., 1985). A paracrine role for glucagon in the regulation of insulin secretion is supported by observations from other groups using mouse models with Gcgr or glucagon deficiencies (Gelling et al., 2003, 2009; Moens et al., 1998; Sørensen et al., 2006).

Our data clearly demonstrate that glucagon can activate the GLP-1R, although with a lower potency compared with its interaction with the Gcgr. The ability of glucagon to make use of the GLP-1R in the absence of functional Gcgrs becomes apparent in

Gcgr ^{β cell^{-/-} mice, where insulin secretion was similar to that of control mice. In *Gcgr*^{-/-} mice, only high doses of glucagon were able to stimulate insulin secretion, again through the GLP-1R. Conversely, without a functional GLP-1R (whether blocked by an antagonist or in the *Glp1r*^{-/-} mouse pancreas), exogenous glucagon still induced a rise in insulin secretion, although the response was slightly reduced compared with that in animals with fully functional GLP-1R and Gcgr.}

A role for pancreatic GLP-1 has been discussed, and studies have suggested local production of GLP-1 in islets (Marchetti et al., 2012; Taylor et al., 2013; Whalley et al., 2011). The original concept was that GLP-1 results from posttranslational processing of proglucagon via PC1/3 in intestinal L-cells, whereas proglucagon in the pancreas is cleaved by PC2, yielding glucagon and the major proglucagon fragment. Nevertheless, studies using Pdx1-Cre to control the expression of *Gcg* and, hence, GLP-1 in the mouse pancreas suggested that pancreas-derived GLP-1 may contribute substantially to the incretin effect following oral glucose administration in mice (Chambers et al., 2017). In contrast, our data demonstrate that the levels of active, fully processed GLP-1(7-36_{NH2}/7-37) in perfused pancreata from control mice are very low and frequently undetectable. Indeed, a recent study based on very sensitive detection methods was unable to identify measurable amounts of GLP-1 in extracts of the normal mouse pancreas (Galsgaard et al., 2018). Hence, under normal circumstances, the secretion of active GLP-1 from the pancreas is negligible. However, we show here that GLP-1R-stimulated insulin secretion can also be elicited by glucagon and, therefore, does not require the presence of locally produced islet GLP-1. Hence, the inhibition of insulin secretion by Ex9, as demonstrated by Chambers et al. (2017) and also evident from our data, may reflect inhibition of glucagon-induced activation of the GLP-1R as well as blockade of excess GLP-1 produced in the context of selective pancreatic reactivation of *Gcg* expression.

The *Gcgr*^{-/-} mouse has massive hyperglucagonemia associated with postnatal enlargement of the pancreas and hyperplasia of the islets, primarily α cells and, to a lesser extent, δ cells (Gelling et al., 2003). Perfusion of *Gcgr*^{-/-} pancreata demonstrated significant levels of active GLP-1, showing that, in conditions with highly increased secretory activity, α cell hyperplasia, and perhaps activation of α cell Pcsk1, bioactive GLP-1 is locally produced. A recent paper showed that, under normal circumstances, islet GLP-1 is not necessary for normal insulin secretion but could play a role during metabolic stress and increased secretory demands (Traub et al., 2017). Further studies are needed to define the conditions enabling processing and secretion of bioactive GLP-1 in islet cells.

The indisputable effect of the GLP-1R becomes obvious in the *Gcgr*^{-/-} pancreas, where we noted an unexpected increase in insulin secretion. However, the combination of excessive glucagon secretion as well as increased levels of active GLP-1 could lead to increased activation of the GLP-1Rs in β and δ cells and, therefore, increased hormone secretion. Notably, GLP-1 blockade through Ex9 in *Gcgr*^{-/-} pancreata completely abolished augmentation of insulin secretion, attesting to the importance of these two receptors for insulin secretion.

Attempting to further elucidate the role of glucagon and activation of the Gcgr without the influence of GLP-1, we used DT-Gcg

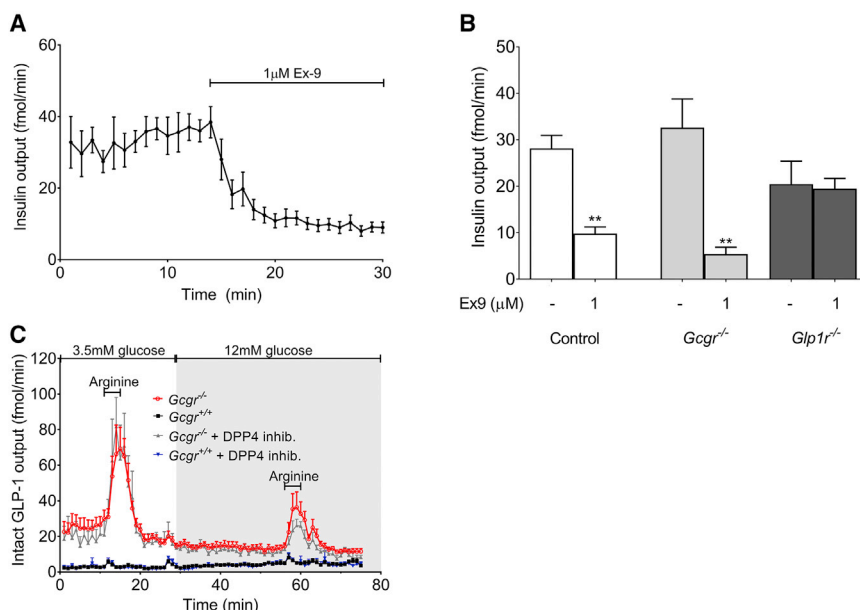


Figure 4. Role of GLP-1 Receptor Inhibition and Intra-islet GLP-1 Secretion in Perfused Mouse Pancreata

(A) Insulin secretion at 12 mM glucose after infusion of Ex9 in control mice.

(B) Mean insulin output was averaged over 10 min before and during addition of Ex9 to the perfused pancreas of control, *Gcgr*^{-/-}, and *Glp1r*^{-/-} mice at 12 mM glucose. Significance was tested by 1-way ANOVA (repeated measures) comparing insulin secretion with and without Ex9 ($n = 4$, ** $p < 0.01$).

(C) Intact GLP-1 (7-36_{NH2}/37) secretion in perfused pancreas from *Gcgr*^{+/+} and *Gcgr*^{-/-} mice with and without a DPP4 inhibitor (ValPyr). Data are represented as mean \pm SEM.

mice with acute knockdown of all proglucagon products. This mouse model has previously been shown previously to have a normal total islet area as well as β cell area compared with control mice (Pedersen et al., 2013). The reduced insulin secretion after glucagon knockdown suggests that glucagon may play a regulatory role in insulin secretion. Glucagon-dependent insulin secretion was only apparent at high glucose levels, indicating that intra-islet glucagon is particularly required at times with high insulin secretion demand. It could be speculated that glucose-stimulated insulin secretion also more generally depends on glucagon levels; thus, when glucagon is low, less insulin is needed to maintain appropriate blood glucose. A stimulating effect of glucagon on insulin secretion at low glucose levels would be inexpedient, if not dangerous, given glucagon's role in the defense against hypoglycemia.

The results from these mouse models with different ways of disrupting glucagon signaling independently support the concept that some degree of local glucagon activity is required for appropriate insulin secretion. Thus, we have demonstrated an important role for glucagon acting directly within the pancreas on the secretion of islet hormones. Furthermore, it is clear that glucagon activates both the glucagon and the GLP-1 receptor on β cells and that experimental inhibition of the effect of glucagon requires simultaneous inhibition of the two receptors, which should be taken into consideration when trying to eliminate glucagon action by only blocking the *Gcgr*. Likewise, the use of GLP-1 receptor antagonists not only blocks the actions of GLP-1 but also any potential action of glucagon on GLP-1 receptors, which should be brought to mind when formulating concepts linking α -cell secretory products to control of α cell function.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.10.018>.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.S. and J.J.H.; Investigation, B.S., O.L., M.B.N.G., and C.B.C.; Resources, D.J.D. and J.J.H.; Supervision, M.M.R., D.J.D., and J.J.H.; Writing – Original Draft, B.S. and J.J.H.; Writing – Review & Editing, D.J.D. and M.M.R.; Funding Acquisition, B.S., D.J.D., and J.J.H.

DECLARATION OF INTERESTS

D.J.D. has served as an advisor, consultant, or speaker for Eli Lilly Inc., Intarcia, Kallyope Inc., Merck Research Laboratories, and Novo Nordisk. J.J.H. has served as a consultant or advisor to Novartis Pharmaceuticals, Novo Nordisk,

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Glucagon	Sigma-Aldrich	G2044
Exendin 9-39	Bachem	4017799
L-arginine	Sigma-Aldrich	A6969
Experimental Models: Cell Lines		
COS-7 cells	ATCC, Virginia, USA	CRL-1651
Experimental Models: Organisms/Strains		
Mouse: DT-Gcg	Pedersen et al., 2013	n/a
Mouse: <i>Glp-1r</i> ^{-/-}	Scrocchi et al., 1996	n/a
Mouse: <i>Gcgr</i> ^{βcell-/-}	Daniel Drucker, University of Toronto	n/a
Mouse: <i>Gcgr</i> ^{-/-}	Gelling et al., 2003	n/a
Recombinant DNA		
Mouse <i>Gcgr</i> DNA	Origene	MC203290
mouse <i>Glp1r</i> DNA	Origene	MC216256
Other		
In-house ELISA for active GLP-1	Vilsbøll et al., 2003	n/a
Glucagon antiserum for glucagon radioimmunoassay	Orskov et al., 1991	4305
Insulin antiserum for insulin radioimmunoassay	Brand et al., 1995	2006-3

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Berit Svendsen (bsvendsen@sund.ku.dk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All experiments were conducted in compliance with animal experiment license (2008/561-1491) issued by the Danish Committee for Animal Research, and approved by the local animal welfare committee at the University of Copenhagen. Mice generated in Toronto were approved by the Mt. Sinai Hospital Animal Care Committee.

Studies were conducted in animals 8-16 weeks of age on a C57BL6/J background, and included sex- and age-matched littermate control mice. Mice were housed up to eight per cage and kept on 12hr light-dark cycle with free access to chow diet and water.

Different mouse strains were used in this study:

- 1) Inducible knockdown of proglucagon-producing cells (DT-Gcg; ([Pedersen et al., 2013](#))) where expression of the human DT receptor is driven by the proglucagon promoter, and DT injections lead to ~90% destruction of proglucagon producing cells. The mice received ip injections of 10 ng DT/g body weight on day 1, 3, and 5.
- 2) Mice with inducible *Gcgr* knockout specifically in β-cells (*Gcgr*^{βcell-/-}) were generated by mating MIP-cre mice ([Campbell et al., 2016](#)) with *Gcgr* flox mice ([Longuet et al., 2013](#)). MIP-Cre (controls) or *Gcgr*^{βcell-/-} mice were treated with 50 mg/kg Tamoxifen ip for 5 consecutive days, then allowed to recover for several weeks prior to use in perfusion studies.
- 3) *Glp1r*^{-/-} mice with global deletion of the GLP-1 receptor ([Scrocchi et al., 1996](#)).
- 4) *Gcgr*^{-/-} mice with global *Gcgr* knockout ([Gelling et al., 2003](#)).

METHOD DETAILS

Cell lines and transfection

COS-7 cells (ATCC, Virginia, USA) were cultured in DMEM 1885 (10% FBS, 2mM glutamine, 180 units/ml penicillin, and 45 g/ml streptomycin (10% CO₂ and 37°C)) and transiently transfected with mouse *Gcgr* or mouse GLP-1 receptor DNA (Origene, Rockville, MA,

USA (MC203290 and MC216256, respectively) using the calcium phosphate precipitation method with addition of chloroquine (Kissow et al., 2012; Luthman and Magnusson, 1983). DNA was diluted in TE-buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) to which 2 M CaCl₂ was added. The DNA-calcium coprecipitate was added to equal amount of HEPES-buffered saline (HBS). Transfection mixture was added to the cells and incubated for 5 h (37°C, 10% CO₂). cAMP assays were performed ~48 h after termination of transfection procedure.

cAMP assay

Transiently transfected COS-7 cells expressing either Gcgrs or GLP-1 receptors were incubated with GLP-1 or glucagon for 30 minutes at 37°C and assayed for cAMP accumulation. To test for antagonism of Ex9, cells were preincubated for 10 minutes with Ex9 followed by incubation with constant concentrations of either GLP-1 or glucagon corresponding to 50%–80% of the maximal cAMP accumulation response (E_{\max}) on the GLP-1 receptor or Gcgr, respectively. Luminescence was measured by Perkin Elmer™ EnVision 2104 Multilabel Reader (Skovlunde, Denmark). All experiments were performed in triplicates and repeated at least three times.

Perfused mouse pancreas

Pancreas perfusions were performed as previously described (Adriaenssens et al., 2016). Briefly, mice were anaesthetized with ip injection of ketamine (90 mg/kg; Ketaminol Vet.; MSD Animal Health, Madison, NJ, USA) and xylazine (10 mg/kg; Rompun Vet; Bayer Animal Health, Leverkusen, Germany). The intestine, spleen, stomach, and kidneys were tied off. The aorta was ligated proximally to the celiac artery, and a catheter was inserted into the aorta providing arterial perfusion with a modified Krebs-Ringer bicarbonate buffer (Adriaenssens et al., 2016). Effluent samples were collected through a catheter in the portal vein. The perfusion system (UP-100 universal perfusion system, Hugo Sachs Elektronik, March-Hugstetten, Germany) maintained constant flow of 1.35 ml/min, perfusion buffer was heated and oxygenated (95% O₂, 5% CO₂) and pressure was monitored throughout the experiment (40–50 mmHg). Experiments exhibiting significant changes in pressure or flow (> 20%) were terminated and discarded.

Experimental protocol

After surgery and perfusion with medium was initiated, the pancreas was allowed to stabilize for 30 minutes.

Protocol a

In a series of experiments, different concentrations of glucagon (0.1, 1, 10nM) were infused for 10 min at 12mM glucose separated by 20 min periods to allow secretion to return to basal level. This protocol was repeated in wild-type, *Gcgr*^{βcell^{-/-}}, *Glp1r*^{-/-}, and *Gcgr*^{-/-} male mice with or without 1μM Ex9 added to the perfusion buffer.

Protocol b

In a second set of experiments, the pancreas was perfused at low glucose (3.5mM) followed by a switch to high glucose (12 mM) in the perfusion buffer. In all experiments, L-arginine (10 mM, Sigma-Aldrich) was infused as stimulus for 5 min periods during both glucose concentrations.

Hormone measurements

Hormone concentrations in the perfusion effluent were measured using in-house radioimmunoassays. Glucagon was measured using a COOH-terminally directed antiserum (4305), which measures fully processed glucagon as well as N-terminally extended molecular forms (Orskov et al., 1991). Insulin was measured using an antibody crossreacting strongly with rodent insulin I and II (2006-3; (Brand et al., 1995)). Intact GLP-1 was measured using a sandwich ELISA for active GLP-1 (Vilsbøll et al., 2003).

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical procedures used and exact values of n are indicated in the figure legends. Responses to arginine and glucagon infusions were evaluated by comparing hormone secretion for 5 or 10 minutes prior to infusion with hormone output during the infusion period using one-way ANOVA analysis. Statistical analyses were performed using GraphPad Prism v.7. Data are expressed as mean ± SEM, and statistical significance was accepted when $p < 0.05$.